FORM PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER	
151.2-US-WO	
U.S APPLICATION NO. (If known, see 37 CFR 1 5)	
09/913927	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE00/00525	19 February 2000 (19.02.00)	19 FEB 1999; 19 FEB 1999

TITLE OF INVENTION: SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND UTILIZATION THEREOF

APPLICANT(S) FOR DO/EO/US: Ulrich Schubert, Peter Henklein, and Victor Wray

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [X] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (unsigned)
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [ ] Other items or information:

JC05 Rec'd PCT/PTO 2 0 AUG 2001

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/913927	PCT/DE00/00525	151.2-US-WO

21. [XX] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1000.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	3 - 20 =	0	x **$18.00**	$	
Independent claims	1 - 3 =	0	x **$80.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1000.00	
[XX] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$ 500.00	
SUBTOTAL =				$ 500.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 500.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 500.00	
				Amount to be refunded:	$
				charged:	$

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0494 . A duplicate copy of this sheet is enclosed.

d. [XX] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO.

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SIGNATURE

Karen S. Canady
NAME

39,927
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Ulrich Schubert et al.	Docket:	151.2-US-WO
Serial No.	To be assigned	International Application No.	PCT/DE00/00525
Filed:	Herewith		
Title:	SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND THE UTILIZATION THEREOF		

CERTIFICATE OF MAILING UNDER 37 CFR 1.10
'Express Mail' mailing label number: EL719114940US
Date of Deposit: August 20, 2001
I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 and is addressed to the Commissioner for Patents, Washington, D.C. 20231.
By: Darlene Ross
Name: Darlene Ross

PRELIMINARY AMENDMENT

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendment:

IN THE SPECIFICATION

Please amend the specification as follows:

At page 1, after the title, please insert the following paragraph:

--This application claims priority from German patent applications number 19908752.0 filed 19 February 1999 and number 19908766.0 filed 19 February 1999.--

At page 17, line 18, please insert the following paragraphs:

--Example 16:

Generation of poly-clonal anti-Vpr-antibodies by immunization of rabbits with sVpr1-96:

5 mg sVpr1-96 was dissolved in water, mixed with complete Freunds adjuvant and was used for standard immunization of rabbits. Serum samples of all bleedings were pooled, aliquoted and frozen, and the antiserum was termed "R-96". In order to establish the lowest sensitivity of R-96 for detecting Vpr, the antiserum was tested in immunoprecipitation using serial dilutions of sVpr1-96, starting from 0.1 to 10 ng. To each dilution 200 µl of human serum derived from a healthy HIV-1 seronegative blood donor was added and the samples were further diluted by addition of 1 mL phosphate buffered saline (PBS) and then subjected to immunoprecipitation with R-96. For this purpose, to each dilution 5 µl of R-96 adsorbed onto 30 µl of Protein-G Sepharose beads (GammaBind-G-Sepharose-beads, Pharmacia LKB Biotechnology, Piscataway, NJ, USA) were added.

Prior to immunoprecipitation with R-96, all dilutions were pre-cleared with Protein-G Sepharose beads loaded with rabbit IgG derived from pre-immune sera. The immune precipitation was conducted using standard conditions as described previously (Schubert and Strebel, 1994). The precipitates were denatured by boiling in SDS gel sample buffer (2% SDS, 1% ß-Mercaptoethanol, 1% Glycerol, 65 mM Tris-hydrochloride (pH 6.8) (10 min, 95°C), separated in 12.5% denaturating SDS-polyacrylamide gel (SDS-PAGE, 12.5% Acryl aide gels, FMC Bioproducts, Rockland, ME) (see Fig. 2B). A dilution of the peptide sVpr1-96 (from 0.01 to 10 nM) was separated in the same gel (Fig. 2A). The separated samples were electrotransfered onto Immobilon polyvenylidene difluoride (PVDF)-Membrane (Millipore Corp., Bredford, MA), the membranes were blocked with 5% BSA in PBS (0,3% Tween 20) and finally incubated with a 1:1000 dilution of R-96 in 3% BSA. Bound antibodies were stained with ^{125}I-Protein G (0.1 mCi/ml; New England Nuclear, DuPont, Wilmington, DE) and visualized by autoradiograpy (Fig. 2). The results of this Western blot analysis demonstrate, that under those conditions 01 ng of sVpr1-96 can be detected in human serum samples using the anti serum R-96. The lowest sensitivity to detect sVpr1-96 by immune precipitation was in the range of 1 to 10 nM sVpr1-96. In other experiments using Western blot as well as immune precipitation techniques (data not shown) show that R-96 also reacts with native viral Vpr, expressed in HIV-1 infected cells.

Example 17:

sVpr1-96 increases virus replication and number of living cells in cultured human PBMCs.

For the purpose of HIV infection, parallel cultures of PBMCs isolated form peripheral blood of HIV-1-seronegative healthy blood donors were stimulated by incubation with phytohaemagglutin (PHA) and interlocking 2 (IL-2) according to previously published methods (Schubert et al., 1995). Activated PBMCs were infected with equal infectious doses of purified virus stocks of the T cell tropic isolates HIV-1NL4-3. The culture was split and one half of the infected culture was treated with 10 nM sVpr1-96, and the other half was incubated with a 10 nM solution of the control peptide, Vpu32-81, which is biologically inert to PBMCs and was synthesized and purified under the same conditions as sVpr1-96 (Henklein et al., 1993). The control peptide Vpu32-81 comprises the 50 amino acid long cytoplasmic domain of the HIV-1 specific virus protein U (Vpu) (Wray et al., 1996). Treatment with peptides was maintained throughout the entire course of the experiment, approximately 80% of the culture medium was replaced every other day with fresh media containing the freshly prepared peptide solutions. For the purpose of estimating the amount of released virus particles, aliquots of cell culture supernatants were frozen at –80°C. At the end of the study, the activity of virus associated reverse transcriptase (RT) was estimated in parallel reactions for each cell culture supernatant sample and plotted against the time in histograms (Figure 3A). Based on those RT-profiles, summarized in Figure 3A, it can be clearly seen that, in the presence of sVpr1-96, an approximately two-fold increase in virus replication occurred during the spread of the infection in the cell culture. This sVpr1-96 induced activation of virus replication begins two days post infection and is maximal at day seven post infection, the peak of virus replication, after then the effect remains constant. Dose dependency revealed that this effect was maximal when sVpr1-96 was applied at a concentration of approximately 10 nM (data not shown). Parallel to the estimation of the RT activities released, the number of live cells was measured in the infection experiments by using a trypan blue exclusion method. This estimation was conducted each time of medium exchange and the numbers of cells were plotted as function of time (Figure 3B). Those results clearly show that, compared to the control culture, the number of living cells is approximately 1.5 fold increased in the presence of sVpr1-96. In general, and also as seen in the control culture (Figure 3B), the spread of infection in HIV-1 infected culture is characterized by a maximum of cytopathic effect at the time of maximal virus replication followed by a drastic decline in the number of living cells. This situation is different in the presence of sVpr1-96, the number of living cell starts

to decline with lower kinetic as in the control culture at day nine post infection. This phenomenon can be explained either by suppression of cytopathic mechanisms like HIV-1 induced apoptosis and/or cell fusion and syncytia formation. Alternatively, sVpr1-96 could merely increase the number of HIV-1 infected cells in the culture. Worth mentioning is the observation that this effect is not restricted to the replication of wild type virus and it also occurred with comparable intensity in PBMCs isolated from different donors. For this purpose, PHA/Il2 stimulated PBL derived from a different donor were infected with T cell tropic wild type virus HIV-1NL4-3 (Figure 3C) or with the chimeric macrophage-tropic virus NL4-3(AD8) (Figure 3D) and treated with 10 nM of sVpr1-96 or the control peptide Vpu32-81. Under the same conditions PBMCs were also infected with the vpu-deficient virus NL(AD8)-UDEL1 (Figure 3E) as well as wit the vpr-deficient virus NL(AD8)deltaR (Figure 3F). It is well known that both, Vpr and Vpu can stimulate virus replication in PBMCs. In both situations and similar to the findings in cultures infected with the wild type virus HIV-1NL4-3 approximately a three to five fold increase in virus replication was observed in the presence of sVpr1-96.

In summary, sVpr1-96 stimulates virus replication in primary human T lymphocytes, and at the same time it causes an increase in the number of living cells. The latter effect occurs independently of the endogenous expression of the viral accessory proteins Vpr or Vpu.

Example 18:

sVpr1-96 complements the replication of vpr-deficient HIV-1-mutants in cultures of primary human monocytes/macrophages.

In order to test the impact of sVpr1-96 on virus replication primary human monocytes/ macrophages "monocyte derived macrophages" (MDM) cells were isolated form blood lymphocytes of three HIV-seronegative healthy individuals (Donors #1 to 3). Cells were differentiated during a 14 day incubation period. Parallel cultures of MDM isolates were infected with equal infectious doses of chimeric macrophage-tropic virus NL(AD8) as well as the isogenic vpr-deficient mutant virus NL(AD8)deltaR. With the start of the infection, parallel cultures were treated with 10 nM sVpr1-96 as well as with 10 nM of the control peptide Vpu32-81. 90% of the culture medium was replaced with fresh medium and peptide solutions every three days. Aliquots of the cell culture supernatants were harvested during a two-month cultivation period and frozen at –80°C.

Finally, the amount of virus associated RT activity was estimated and plotted as a function of time, the corresponding replication profiles are demonstrated in Figure 4. While wild type virus NL4-3(AD8) was able to establish a productive infection in MDM culture with maximum of virus replication on day 24 post infection the replication and spread of infection in cultures infected with the vpr-deficient mutant NL(AD8)deltaR was significantly reduced (Figure 4A). In MDM cultures derived from donors #1 and #2 replication of the vpr-deficient mutant NL(AD8)deltaR was approximately only 15% of the replication of the wild type virus (Figure 4B,C). In the MDM culture derived from donor #3 for NL(AD8)deltaR even no productive infection at all was detectable (Figure 4D). The reduced replication vpr-deficient mutant NL(AD8)deltaR is consistent with the previously described function of the accessory function of Vpr in macrophages/monocytes. However, continuous addition of 10 nM sVpr1-96 to the cell culture medium during the entire course of infection did not positively affect the replication of the wild type virus NL4-3(AD8), the replication profile was even reduced to approximately ~ 60% of the non treated culture (Figure 4A). In contrast, addition of 10 nM sVpr1-96 stimulated significantly replication of the vpr-deficient mutant NL(AD8)deltaR: the virus production in MDM cultures derived from 3 different donors was enhanced by the presence of sVpr1-96 in the culture medium. The addition of exogenous peptide sVpr1-96 thus rescued virus replication of vpr-deficient viruses to near wild type levels as demonstrated by the replication profiles in the presence of the peptide (Figure 4C, D). Even in MDM cultures that had no detectable spread of infection for the vpr-deficient mutant NL(AD8)deltaR, addition of exogenous peptide sVpr1-96 completely restored virus replication to wild type levels (Figure 4D).

In summary, while exogenous peptide sVpr1-96 has no positive effect on the replication of wild type virus in cultured human monocytes/macrophages, it can complement the accessory function of endogenous Vpr in vpr-deficient virus mutants and thus restore virus replication competence of those mutants to near wild type levels.

Example 19:

1H NMR-Spectroscopy on sVpr1-96

First NMR spectra of sVpr1-96 -were recorded in 2mM peptide solution (17mg/mL) in 50% (w/v) tri fluoro ethanol (TFE). The 1D spectra (shown as abscissa and ordinate in Figure 5) demonstrates relatively strong line broadening that is quite unusual for this small 96 amino acid protein, providing evidence that, under the solution conditions, the peptide sVpr1-96 at least partially may have the tendency to undergo self-association. The peptide was also dissolved in aqueous systems without any salt or buffer, the addition of dithiothriethiol (DTT) had no detectable effect on the spectra, thus excluding the formation of disulfide bounds. The NMR data, however, do not provide clear evidence for the existence of high order oligomeric structures above 100 kDa, as demonstrated previously using different techniques (Zhao et al., 1994). In case such oligomeric structures (presumably in the order of hexamers) of sVpr1-96 would exist, such complexes would tumble very slowly and cause broad signal in the spectra. However, such a phenomenon was not detectable in the spectra recorded for sVpr1-96. Individual spin systems could be identified, for instance three times alanine and one time for valine. As those spin systems are distributed throughout the entire molecule, this observation is also inconsistent with the existence of the peptide as a hexamer. Those preliminary data are rather indicative for the presence of sVpr1-96 in a steady state between monomeric and dimeric structures, both of which tend to induce signal broadening.

In order to further solve the problem of line broadening, 2D TOCSY and NOESY spectra sVpr1-96 were recorded that altogether brought evidence for the presence of cis/trans isomeric form in the molecule: certain parts of sVpr1-96 exhibit extra intensive broad signals while sharp cross peaks were identified for another regions of the molecule in the 2D spectra. In the lower field of the TOCSY spectra (10-9.3 ppm), three signals corresponding to tryptophan side chains were identified (Figure 5A). Further enlargement of those cross peaks provided additional insight into the heterogeneity of those signals; beside the main signal there are at least two minor signals. The same observations were made for histidine (Figure 6B) and arginine (Figure 6C). These types of amino acid side chains are distributed over the entire length of the molecule sVpr1-96 (tryptophan in positions 18, 38 and 54; histidine in positions 33, 40,45, 71 and 78, and arginine in positions 12, 32, 36, 62, 73, 77, 80, 87, 88 and 90). Furthermore, those cross peaks represent very sharp signals. Consequently, it could be assumed that the observed phenomenon, particularly the relatively broad

lines in the 1D-spectra and the signal multiplication in 2D spectra is not the result of oligomerization as reported previously for Vpr (Zhao et al., 1994).

The individual enlargement of 2D spectra shown in figure 6 point to the relative existence of approximately 20% heterogeneity within the investigated signals that, all together, is typical for cis/trans isomerism caused presumably by the four proline residues in sVpr1-96. Such isomers represent an unusually high cis-content (up to 40%) of proline residues in positions 14 and 35, that could be attributed to the close vicinity of aromatic amino acid side chains to those proline residues.

In order to further investigate the so far first observation of cis/trans isomerism in sVpr1-96, the following short peptides were synthesized and analyzed according to the invention:

<u>sVpr1-20</u>
(1M - E - Q - A - P - E - D - Q - G - P10 - Q - R - E - P - Y - N - E - W - T - L20), and
<u>sVpr21-40</u>
(21E - L - L - E - E - L - K - S - E - A30 V - R - H - F - P - R - I - W - L - H40).

In the investigated 1D and 2D NMR spectra, multiple signals corresponding to different proline residues were identified for both peptides, sVpr1-20 sVpr21-40. For the peptide sVpr21-40, approximately 10% of the molecule was found in the cis conformation. Similar results were obtained for the peptide sVpr1-20 with three different cis proline structures accounting together up to 30% of the entire molecule being in cis conformation.

In summary, it was observed that cis/trans isomerism of proline residues are the major cause for the heterogeneous structures of the sVpr1-96 full length molecule. Based on the NMR calculation of the short peptides it can be predicted that for the entire molecule sVpr1-96 approximately 40% single cis, 6% double cis, and 0,4% tipple cis conformation of all proline residues may exist, while only 59% of all proline residues exist in trans conformation.

In a further step of the procedure, the proline residues are identified which contribute primarily to the observed heterogeneity. Those proline residues are exchanged in sVpr1-96 by asparagine, a

conservative amino acid exchange. The aim is to replace proline with amino acid side chains that have a similar impact on the folding of the protein backbone but do not participate in the cis/trans isomerism. The exchange of proline to asparagine is based on previously published studies on the structure correlation matrix between amino acid side chains and protein folding (Livingston and Barton, 1996). Such constructed Vpr mutants that cannot undergo cis/trans isomerism are the ideal substrate for structural analyses using NMR and x-ray crystallography. The biological activity of such proline to asparagine mutated Vpr molecules can also be established.

Example 20:

Materials and Methods.

Example 20a:

Molecular HIV-1 clones and plasmid construction.

For the construction of T cell tropic viruses based on the molecular clone HIV-1NL4-3, the previously published plasmid pNL4-3 was used (Adachi et al., 1986). For the construction of macrophage-tropic viruses, the previously published plasmid pNL4-3(AD8) was used (Schubert et al., 95; Freed et al., 1995; Freed and Martin, 1994). This plasmid encodes the molecular infectious DNA of a macrophage-tropic virus that carries the env gene of the primary macrophage-tropic virus AD8 (Theodore et al., 1995) inserted into the back bone T cell tropic viruses HIV-1NL4-3 (Freed and Martin, 1994). For the replication competence of HIV-1 in monocytes and macrophages, a domain of the env gene is necessary, including the V3- loop (Schubert et al., 95; Freed et al., 1995; Freed and Martin, 1994; O'Brien et al., 1990; Shioda et al., 1991). For the construction of vpr-deficient mutant pNL(AD8)-deltaR, the plasmid pNL4-3(AD8) was linearized with the restriction enzyme EcoRI at base pair position 5743, the overhanging ends were filled in with DNA-polymerase I and the plasmid was then religated. This procedure causes a frame shift mutation within the vpr reading frame resulting in the expression of a N-terminal fragment of Vpr, which is unable to perform any of the so far described biological activities of Vpr.

Example 20b:

Cell culture.

Hela cells were cultivated in Dulbecco's' modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. PBMCs ("peripheral blood mononuclear cells") were isolated by gradient

centrifugation of blood lymphocytes of healthy HIV-1-seronegative individuals, and cells were aliquoted and frozen in liquid nitrogen. Two days prior to infection, PBMC cultures were stimulated with phytohaemagglutin (PHA, 1mg/ml) and human interleukin-2 (hIL-2, 20 U/ml). The treatment with hIL-2 was continued throughout the entire course of the experiment. MDM ("monocyte-derived macrophages") were isolated by counter current elutriation using standard and previously published methods (Schubert et al., 1995; Ehrenreich et al., 1993). Adherent cultures of MDM were pre-cultured in DMEM supplemented with glucose (4.5 g/L), penicillin (50 U/ml), streptomycin (50 mg/ml), L-glutamine (2mM), sodium pyruvate (1mM), and 10% human serum using a modified method described by Lazdins et al. (1990). Following a two week period of differentiation, MDM were resuspended, harvested, and re-plated at a concentration 0.5 x 10^6 cell per ml, and further incubated for approximately another two to three days.

As a control for potential contamination with CD4+ T lymphocytes -parallel cultures of MDM from each were incubated with the T cell tropic virus HIV-1NL4-3. In all experiments, no infection HIV-1NL4-3 could be established in any of the MDM cultures used in those experiments attesting for the absence of CD4+ T lymphocytes in our MDM preparation.

Example 21:

Transfection and generation of virus stocks.

For the purpose of generating virus stocks, plasmid DNA of molecular HIV-1 clones were transfected in HeLa cells using calcium phosphate precipitation. Confluent cultures of HeLa cells ($5x10^6$ cells) were transfected with 25 µg plasmid DNA associated with calcium phosphate crystals produced according to the method described previously by Graham and van der Eb (1973). Cells were incubated and subsequently subjected to a glycerol shock according to a method described previously by Gorman et al. (1982). For production of concentrated virus stocks, cell culture supernatant of the transfected cultures were harvested two days after transfection. Cells and debris were separated by centrifugation (1,000 x g, 5 min, 4°C) and filtration (0.45 µm pore size). Virus particles were pelleted by ultra centrifugation (Beckman SW55 Rotor, 1.5 hr, 35,000 rpm, 10°C) and resuspended afterwards in 1 ml of DMEM Medium. Virus stocks were sterilized by filtration (0.45 µm pore size), aliquoted, and frozen at –80°C. Individual virus stock standardized by estimation of

the amount of RT (reverse transcriptase) activity using a previously described assay (Willey et al, 1988) based on the incorporation of [32P]-TTP into an oligo(dT)-poly(A) template.

Example 22:

1H NMR on sVpr peptides

1D and 2D 1H NMR spectra were recorded on a DMX 600 Bruker NMR-Spectrometer without spinning at 300°K. Spectra were calibrated based on the proton in TFE at 3.95 ppm.

More details are given in the figure legend. –

IN THE CLAIMS

Please cancel claims 1-30 without prejudice to Applicants' right to present the subject matter of these claims in a subsequent amendment or application, and add new claims 31-33 as follows:

31. (NEW) A synthetic peptide comprising a regulatory virus protein R (Vpr) of the human immunodeficiency virus type 1(HIV-1).

32. (NEW) The synthetic peptide of claim 1, comprising:

(a) a 96 amino acid Vpr protein ($sVpr^{1-96}$);

(b) a 47 amino acid N-terminal peptide ($sVpr^{1-47}$);

(c) a 49 amino acid long C-terminal peptide ($sVpr^{48-96}$); or

(d) a. fragment of at least approximately 15 amino acids of any one of (a)-(c).

33. (NEW) The synthetic peptide of claim 32, wherein the fragment comprises $sVpr^{1-20}$ or $sVpr^{21-40}$.

REMARKS

The above preliminary amendment is made to introduce the amendment filed under PCT Article 34 in the WIPO application. The subject matter of this amendment to the specification is supported by the figures and figure legends of the application as originally filed.

In addition, Applicants have amended the claims to present them in a format more suitable for United States practice. These amendments to the claims were not required to distinguish the claimed subject matter over the prior art or for other reasons of patentability.

Applicants respectfully request entry of the preliminary amendment described herein prior to calculation of the filing fee and prior to examination and consideration of the above-identified application.

If a telephone conference would be helpful in resolving any issues concerning this communication, please contact Applicant's undersigned attorney.

Respectfully submitted,

GATES & COOPER LLP
Attorneys for Applicant(s)

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Date: 20 August 2001

By: _____
Name: Karen S. Canady
Reg. No.: 39,927

KSC/dr
G&C 151.2-US-WO

SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND THE UTILIZATION THEREOF

Summary:

The invention pertains to synthetic (*s*) peptides derived from the viral regulatory protein R (Vpr) of the human immunodeficiency virus type 1 (HIV-1), particularly the chemical synthesis of the 96 amino acid full length Vpr protein, $sVpr^{1-96}$, as well as several fragments thereof. The application of those synthetic HIV-1 Vpr peptides in biological assays, for molecular and structural characterization, as well as for the development of anti-Vpr antibodies and serodiagnostic test systems are disclosed.

So far the only *in vitro* characterized biochemical activity of HIV-1 Vpr is that of a cation-selective ion channel (Piller *et al.*, 1996, list of references at the end of examples). Those studies are based on the assumption that the C-terminal alpha-helix (amino acid position 46 to 71 in Vpr), which contains certain similarity to the honey bye poison melittin, has the capability to govern as a transmembrane anchor the formation of a membrane pore. Indeed, recombinant Vpr expressed in *Escherichia (E.) coli* was reconstituted in artificial planar lipid bilayers. Using this system, an ion channel activity was identified that is gated by the membrane potential. The gating of the channel is dependent on the positively charged C-terminal domain of Vpr which is believed to interact with the cytoplasmic part of the cell membrane.

There is evidence that Vpr forms homo oligomers: a recombinant Vpr-fusion protein was found in oligomeric structures of a molecular weight >100 kDa (Zhao *et al.*, 1994b). So far, this observation could not be confirmed by studies on viral Vpr. The molecular structure of Vpr was investigated by two laboratories using analyses of the secondary structure on short Vpr-Peptides: alpha-helical regions in Vpr positions 50-

82 was identified by NMR-studies on overlapping peptides in aqueous trifluorethanol (TFE) as well as in sodium dodecylsulfate (SDS)-micelles (Yao *et al.*, 1998). The propensity for helix formation was predicted by several authors for regions within the C-terminus as well as the N-terminus of Vpr (Mahalingam *et al.*, 1995a-d; Yao *et al.*, 1995; Wang *et al.*, 1996b). Recent studies on 25 amino acid long peptides using CD-spectroscopy in aqueous TFE solutions (Luo *et al.*, 1998) provided first experimental evidence for the existence of N- and C-terminal helices in Vpr. Based on mutational analyses, numerous and, at least partial, contradictory information was reported that tried to correlate certain primary and secondary structures of Vpr with different biological activities reported for Vpr (Mahalingam *et al.*, 1995a-d, 1997; Wang *et al.*, 1996a,b; Nie *et al.*, 1998; Di Marzio *et al.*, 1995).

The chemical total synthesis of a Vpr peptide was first described 1997 by Rocquigny and colleagues. The authors reported the synthesis of a 96 amino acid long peptide derived from the virus isolate HIV-$1_{89.6}$ (Collman *et al.* 1992). Beside the disadvantages of this synthesis reported by the authors in their publication (see in the further text) this protein is different in nine amino acid positions compared to the Vpr protein derived from the virus isolate HIV-1_{NL4-3}, the synthesis of which is described for the first time in the present specification. As such, there is a 10% difference in the amino acid sequence between the already described (Rocquigny *et al.*, 1997) and the synthetic products covering the entire as well as partial sequences of the Vpr protein derived from the virus isolate HIV-1_{NL4-3} (Adachi *et al.*, 1986) as described in detail in the present processes.

Rocquigny and colleagues (1997) did not reveal any information about the purity and the molecular characteristics of those synthetic Vpr peptides. The authors merely described far-Western blot techniques that demonstrate binding of SDS-denatured Vpr peptide with the viral nucleocapsid protein $p7^{NC}$ derived from the same HIV-1-isolate. So far, this observation of $p7^{NC}$ - Vpr - interaction could not be repeated by

any other of the numerous laboratories working worldwide in Vpr research. An important disadvantage of the Vpr synthesis described by Rocquigny and colleagues (1997) is the fact that none of the so far well-characterized biological functions of HIV-1 Vpr could be demonstrated by the authors for those synthetic peptides. Specifically, the authors show that this particular Vpr peptide does not bind to $p6^{Gag}$, a well recognized characteristic of native viral Vpr (Paxton *et al.,* 1993; Lavallee *et al.*, 1994; Kondo *et al.*, 1995; Lu *et al.*, 1995; Kondo and Göttlinger, 1996). In addition, the authors report that this Vpr peptide does not form oligomeric structures, and there are some indications that this synthetic product is insoluble in aqueous solutions. A model of Vpr - $p7^{NC}$ - interaction was introduced by the same laboratory in an additional study based on structural analyses conducted on partial sequences of Vpr peptides, however, no detailed information about structural and experimental data were provided in this or other reports published by the authors (Roques *et al.,* 1997).

Partial sequences of synthetic Vpr peptides (amino acid positions 50 to 75, 50 to 82, and 59 to 86) were used for NMR studies (Yao *et al.*, 1998). Another group applied circular dichroism spectroscopy to investigated two 25 amino acid long peptides derived from the predicted alpha helical domains in Vpr (Luo *et al.*, 1998). Furthermore, short approximately 20 amino acid long peptides derived from the C-terminal region of Vpr comprising the motif "HF/SRIG" at a concentration range of 0.7 to 3 micro-M had cytotoxic activity towards different yeast strains, for example *Saccharomyces cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe* (Macreadie *et al.*, 1996, 1997). Elevated concentrations of bivalent cations, especially magnesium and calcium, prevented uptake and thus the toxic effects of Vpr-peptides. Continuing studies provided evidence that the C-terminal Vpr peptide (amino acid positions 71-82) can induce permeabilization of membranes, the reduction of membrane potential, and eventually cell death in $CD4^+$ T cells (Macreadie *et al.*, 1997). Similar toxic effects were also for full length (Arunagiri *et al.*, 1997). For those studies the same recombinant glutathione S-transferase (GST) - Vpr - fusion

protein was used which was also employed for ion channel studies on Vpr before (Piller *et al.*, 1996). Nevertheless, as in previous studies the authors reported problems with solubility of the recombinant product in aqueous systems.

Recombinant Vpr derived from the viral isolate HIV-1_{NL4-3} was expressed in insect cells infected with recombinant baculoviruses (Levy *et al.*, 1995). The purification of those products was merely conducted by immune affinity chromatography on immobilized polyclonal antibodies directed against the N-terminal domain of Vpr. For this procedures cell culture supernatants were applied as recombinant Vpr was secreted into the culture medium.

Strategies for large scale production of recombinant Vpr have not been described thus far. In most cases, cell culture supernatants containing recombinant Vpr were used for biological assays. In such an assay it was shown that recombinant Vpr activates virus replication in PBMC (peripheral blood mononuclear cells) as well as in several monocyte and T cell lines latently infected with HIV-1. Significant disadvantages of these already described methods are:

- low yield that does not allow production of mg-amounts of highly purified Vpr products;
- detergents were added to the recombinant Vpr during the process of affinity purification that required subsequent dialysis and renaturation;
- no studies about the potential of post translational modification of Vpr in insect cells were reported.

Expression, purification and biochemical characterization of recombinant Vpr was first described 1994 by Zhao and colleagues. For this procedure the coding sequence of Vpr protein derived from the virus isolate HIV-$1_{89.6}$ was expressed in *E. coli* as a fusion protein. For the purpose of purification of the recombinant product, the 25 amino acid FLAG epitope was fused on the C-terminus. Besides oligomerization, no

biological activity was reported for this recombinant product. A significant disadvantage of this method is the fact that Vpr is not expressed in its authentic sequence, but as a fusion protein.

In another procedure, Vpr protein derived from the virus isolate HIV-1_{HXB2} was expressed in *E. coli* as a GST - fusion protein (Piller *et al.*, 1996). After affinity chromatography on glutathione – agarose, Vpr was released from the fusion protein by proteolytic cleavage with thrombin. A significant disadvantage of the method is the fact that Vpr after thrombin cleavage tends to aggregate and could not be sustained in aqueous solution. It was reported by Arunagiri and colleagues (1997) that Vpr produced with this method could not be maintained in aqueous solution without protein precipitation and aggregation following cleavage of the GST fusion part, while only the GST - Vpr fusion protein was usable for test systems in aqueous solutions.

The patent application WO 95/26361 (Azad, A.A., Macreadie, I.G., Arunagiri, C., 1995) describes biologically active peptide fragments of HIV Vpr proteins; pharmaceutical compounds that contain those peptides or biologically active analogs thereof; antagonists of Vpr-peptides as well as pharmaceutical compounds that contain such Vpr - antagonists. The chemical synthesis of full length Vpr is not described in this method.

The patent application WO 96/07741 (Cohen, E.; Bergeron, D.; Checroune, F.; Yao, X.-J.; Pignac-Kobinger, G., 1996) protects chimeric molecules consisting of Vpr from HIV-1 and Vpx from HIV-2 that are specifically incorporated into HIV-1/HIV-2 virus particles and there interfere with the structure and function of budding virions Those chimeric molecules are protected for the application in gene therapy of HIV-1/HIV-2 infections.

The patent application WO 96/08970 (Weiner, D.B.; Levy, D.N.; Refaeli, Y., 1996) describes methods to block cell division lymphocyte activation using Vpr proteins, fragments of Vpr or sequences of vpr genes. The chemical synthesis of Vpr proteins is not described in this method.

The application of *vpr* genes in screening assay for anti - HIV - pharmaceuticals is described in US patents 5721104 and 5639619, for determination of HIV-2 infection in US patent 5580739, a Vpr-receptor -protein is described in US patent 5780238.

The invention is based on the need to develop a protocol for the high yield synthesis of Vpr proteins in mg-amounts, the purification of those Vpr proteins, and so that the end product, the highly purified Vpr proteins, can made available for general usage.

According to the invention, the problem is solved by the provision of the protein sVpr$^{1-96}$ as well as the following peptides:

- a forty seven amino acid long N-terminal peptide (sVpr$^{1-47}$),
- a forty nine amino acid long C-terminal peptide (sVpr$^{48-96}$) and fragments of those peptides thereof, for example:
- overlapping approximately fifteen amino acid long peptides for the purpose of epitope mapping and isoelectric focusing;
- approximately twenty amino acid long peptides for the structural and functional characterization of individual domains in Vpr, particularly the peptides sVpr$^{1-20}$ and sVpr$^{21-40}$:

sVpr$^{1-96}$:

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln

- His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser-OH

$\underline{sVpr^{1-47}}$:

H-Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr-NH_2

$\underline{sVpr^{48-96}}$:

Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser-OH

$\underline{sVpr^{1-20}}$ as mutant $\underline{sVpr^{1-20}(Asn^{5,10,14})}$:

H-Met - Glu - Gln - Ala - Asn - Glu - Asp - Gln - Gly - Asn - Gln - Arg - Glu - Asn - Tyr - Asn - Glu - Trp - Thr - Leu-NH_2, and

$\underline{sVpr^{21-40}}$ as mutant $\underline{sVpr^{21-40}(Asn^{35})}$:

H-Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Asn - Arg - Ile - Trp - Leu - His-NH_2 ,

fragments of those peptides comprising approximately fifteen amino acid long peptides,

$sVpr^{11-25}$:

H-Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu-NH_2,

$sVpr^{41-55}$:

H-Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala-NH_2,

$sVpr^{46-60}$:

H-Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile-NH_2,

$sVpr^{56-70}$:

H-Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile-NH_2,

$sVpr^{66-80}$:

H-Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg-NH_2,

$sVpr^{76-96}$:

H-Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser-OH.

The C-terminal Vpr-peptide was synthesized on a serine resin using a Perkin - Elmer - peptide synthesizer. All N-terminal peptides were synthesized on a polystyrene - polyoxyethylen -resin. The chain elongation was performed using the FMOC (Fluormethyloxycarbonyl)-strategy using certain protection groups. At the end of the synthesis the cleavage of the protection groups was performed using a cleavage

solutions consisting of 95% trifluoro acetic acid (TFA), 3% triisopropylsilane and 2 to 5 % ethanedithiol, depending on the peptide length synthesized. The resin was removed, the reaction mixture was concentrated and heptane was added. Following concentration the remaining oil was digested with diethyl ether. The raw peptide was removed and lyophilized in acetic acid. Purification of the raw peptide was performed on a preparative HPLC - system (High Pressure Liquid Chromatography). All peptides were purified on a column of silica gel using a linear gradient of TFA and water in acetonitrile. The eluated peptides were concentrated and lyophilized.

Surprisingly, it was found that in contrast to previously described recombinant and synthetic Vpr products, even at mM concentration *s*Vpr - peptides are very soluble in water and remain stable without any sign of protein aggregation and protein precipitation following the described purification protocol in accordance with the invention.

It was demonstrated that the peptide *s*Vpr$^{1\text{-}96}$ adopts a folded structure, is immunologically reactive and possess biological activities comparable to native viral Vpr.

For the first time the chemical synthesis of Vpr - proteins and fragments thereof are described which comprise amino acid sequence of the virus isolate HIV-$1_{NL4\text{-}3}$. The term synthetic (*s*) Vpr - peptides within the scope of the present specification illustrates those peptides synthesized by solid phase peptide synthesis which comprises the authentic amino acid sequence of the native Vpr-Proteins encoded by the *vpr* gene derived from the molecular virus isolates HIV-$1_{NL4\text{-}3}$.

The essence of the invention lies within the combination of already know characteristics (starting materials, synthesis resin, peptide synthesizer) and novel solutions, the first chemical synthesis of those compounds, the synthesis strategy, the

specific protection groups, the cleavage resin trifluoroacetic acid-triisopropylsilane-ethanedithiole according to the invention, the application of certain gradients of solvents (TFA - water : TFA - acetonitrile) for the purpose of purification of peptides, - which are mutually influential and result in their entire action in an advantage of use and the desired success, in that synthetic sVpr-peptides are now available.

The according to the invention synthesized peptides are characterized by the following uniqueness:

They are extremely well soluble in aqueous systems enabling for the first time peptide concentrations as high as in the mM concentration range. This in turn is an essential prerequisite for following structural analysis using NMR (nuclear magnetic resonance)-spectroscopy and X-ray crystallography.

The peptides can be produced in mg amounts under economically reasonable conditions and can be purified to the highest standard. The biological characteristics and immunological reactivity of the peptides are identical to that of the native viral Vpr proteins. The peptides can be used for a variety of applications in the basic research as well as in the applied research in areas of HIV virology.

The peptides according to the invention are used in biological assays, in structural analyses of Vpr and domains thereof, for the generation of antibodies directed against HIV peptide sequences, in anti-viral reagents, for the generation of test systems for the screening of potential Vpr inhibitors, for establishment of cell culture and animal models, for the investigation of mechanisms of Vpr in HIV pathology, for *in vitro* assembly of HIV, for generation of novel vectors in gene therapy, and for the development of serological assays, specifically a Vpr capture ELISA (enzyme linked immune solvent assay).

The created products according to the invention can be used for the determination of the molecular structure of Vpr using NMR- and CD spectroscopy as well as X-ray crystallography. Those structural information in turn are essential for understanding the molecular mechanisms of Vpr proteins in the HIV replication cycle and their role in pathological mechanisms involved in AIDS related diseases. Furthermore, those products can be used for the development and the design of high throughput *in vitro* test systems to search for potential Vpr inhibitors as well as for the generation and characterization of Vpr specific antibodies and serological test systems.

The invention will be used in areas like peptide chemistry, basic research in virology, structural analyses, and medical diagnosis. The invention can be used for the generation of poly- and monoclonal Vpr specific antibodies, specifically for the generation of epitope different Vpr specific antibodies. Further areas of application are: serological test systems, specifically Vpr antigen (Ag) ELISA, as standard antigen for calibration of Vpr - Ag ELISA -techniques, for detection and quantitation of viral Vpr in blood samples of HIV infected individuals, for test systems that characterize Vpr inhibitors, for complementation of the function of endogenous viral Vpr in cultured cells infected with *vpr*-deficient HIV mutants, for complementation of the function of viral Vpr in cultures of human lymphocytes infected with *vpr*-deficient HIV-mutants and for complementation of the function of viral Vpr in cultures of differentiated human monocytes / macrophages infected with *vpr*-deficient HIV-mutants.

The invention can be used for the characterization of reagents that :

a) block the interaction of Vpr with cellular factors, like for the glucocorticoid-receptor, transcription factors and other DNA interacting enzymes and factors;

b) regulate or block the transcription-activating function of Vpr and the activity of Vpr on steroid hormones;

c) regulate or block the transport of Vpr alone, or in conjunction with components of the HIV-pre-integration complex, and the incorporation of Vpr into budding virions during virus assembly;

d) regulate or block the Vpr-induced cell cycle arrest, and the effect of Vpr on cell differentiation and cell growth;

e) regulate or block the cytotoxic effect of Vpr, and

f) regulate or block the ion channel activity of Vpr.

Furthermore, the invention allows the application in the development and design of *in vivo* test systems for the characterization of Vpr inhibitors and animal studies.

Another advantage is that with this invention for the first time concentrated solution of Vpr can be generated for molecular, structural and function analyses necessary for the design of Vpr specific inhibitors. Another application of the invention is the reduction of the flexibility of Vpr's N-terminus using structure stabilizing factors like the UBA2-domain of the DNA repairing enzyme HHR23A which binds to Vpr, Fab-fragments derived from Vpr-specific immune globulins or viral factors, specifically components of the HIV-1 Gag polypeptide precursor $Pr55^{Gag}$ which interact with Vpr during virus assembly, the human glucocorticoid receptor or components thereof. The invention support studies on the *in vitro* assembly of retroviral pre-integration complex, the development of *in vitro* and/or *in vivo* applicable methods of gene transfer, DNA transfection, integration into chromosomal and episomal host DNA, or other methods of gene transfer into cells, tissues or complete organisms with the purpose of gene therapeutic application.

The following Examples serve to explain the invention, without being limited thereto.

Examples

Example 1:

Synthesis of Vpr-peptides - general protocol

Synthesis of the C-terminal Vpr-peptides was conducted using a ABI 433A synthesizer (Perkin Elmer) and a serine-resin provided by the company Fa. Rapp Polymere, Tübingen, Germany. All N-terminal Vpr peptides were synthesized on a polystyrene-polyoxyethylen-resin, "TentaGel R-RAM-resin" provided by the company Fa. Rapp Polymere, Tübingen, Germany. Synthesis of peptides was performed using the FMOC (fluoromethyloxycarbonyl)-strategy using the following protection groups: O-t.butylester for glutamate and asparagine, OtBu-ether for serine, tyrosine and threonine, Boc (tert-butoxycarbonyl-) for lysine and tryptophan, Trt (trityl - triphenylmethyl-) for histidine, glutamine and asparagine, and Pbf (2.2.4.6.7-pentamethyl- dihydrobenzofuran-5-sulfonyl-) for arginine. After finishing the synthesis cleavage of the protection groups was conducted using a mixture consisting of 95% trifluoracetic acid, 3% triisopropylsilane, and 2 to 5 % ethanedithiol, depending on the specific peptide sequence. The resin was removed, the reaction mixture was concentrated and heptane was added. Following concentration the remaining oil was digested with diethyl ether. The raw peptide was removed and lyophilized in 10% acetic acid.

Example 2:

Purification of peptides - general protocol:

100 mg of the raw peptide was purified by preparative HPLC using the Shimadzu LC-8 system. All peptides were purified on a column (300 x 400 mm Vydac-RP18-Säule, grain size 15 - 20 μM) containing column of silica gel. A linear gradient consisting of 1% TFA (trifluor acetic acid) in water 0,1% TFA in 80% acetonitrile was applied with a flow rate of 100 ml / min. Eluted peptides were concentrated and lyophilized.

Example 3:

$sVpr^{1-96}$

The peptide was synthesized on a TentaGel S-AC-resin (0.20 mmol/gram) using an ABI 433 synthesizer. At the end of the synthesis procedure, FMOC-protection groups were cleaved off and the resin was washed first with dimethylformamide and methylenchloride and then dried. The peptide was removed from the resin and purified as described above.

molecular weight: calculated: 11378 found: 11381

H - Met-Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala -

Ser - Arg - Ser – OH.

Figure 1: sVpr$^{1-96}$ - direct separation in SDS-PAGE (A);
immune precipitation prior to SDS-PAGE (B).

Figure 2: sVpr$^{1-96}$ - preparative purification of the raw - HPLC-chromatogram.

Figure 3: sVpr$^{1-96}$ - mass spectrum (% int. and molecular weight).

Example 4:

sVpr$^{1-47}$

in analogy to examples 1 to 3.

molecular weight: calculated: 5728 found: 5728.8

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu

- Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr - NH_2.

Figure 4: sVpr$^{1-47}$ - mass spectrum (% int. and molecular weight).

Example 5:

sVpr$^{48-96}$

in analogy to examples 1 to 3.

Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser – OH.

Example 6:

sVpr$^{1-20}$

in analogy to examples 1 to 3.

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - NH_2.

Figure 5: sVpr$^{1-20}$ - mass spectrum (% int. and molecular weight) (%Int. 10% =111 mV[sum= 9505 mV].

Example 7:

sVpr$^{1-20}$(Asn^{5,10,14})

in analogy to examples 1 to 3.

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - NH_2.

Example 8:

sVpr$^{21-40}$

in analogy to examples 1 to 3.

Wildtype-sequence:

H - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Asn - Arg - Ile - Trp - Leu - His - NH_2.

Figure 6: sVpr$^{21-40}$ - mass spectrum (%Int. 10% =335 mV[sum= 28541 mV]).

Example 9:

sVpr$^{21-40}$(Asn^{35})

in analogy to examples 1 to 3.

H - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Asn - Arg - Ile - Trp - Leu - His - NH_2.

Example 10:

sVpr$^{11-25}$:

in analogy to examples 1 to 3.

H - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - NH_2.

Example 11:

sVpr$^{41-55}$:

in analogy to examples 1 to 3.

H - Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - NH_2.

Example 12:

sVpr$^{46-60}$:

in analogy to examples 1 to 3.

H - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - NH_2.

Example 13:

sVpr$^{56-70}$:

in analogy to examples 1 to 3.

H - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile-

NH_2.

Example 14:

sVpr$^{66-80}$:

in analogy to examples 1 to 3.

H - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - NH_2.

Example 15:

sVpr$^{76-96}$

in analogy to examples 1 to 3.

H-Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser – OH.

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Figure legends:

Figure 1: Structural and functional domains in Vpr

The following primary and secondary structural motifs are aligned to the amino acid sequence of the Vpr protein derived from the Isolate HIV-1_{NL4-3}: The negatively charged N-terminus (label (1), positions 1 - 17); helix alpha-1 (Label (2), positions 18 - 37); a not further defined region (label (3), positions 38-51); helix alpha-2 (label (4), positions 51 - 76); a positively charged C-terminus (label (8), positions 77 - 96). Overlapping to labels (1) to (5) and (87) the following domains are indicated: a leucine- and isoleucine-rich regions termed as "leucine - zipper - like or "LR-domain" (label (5), positions 60 - 80); a region containing the repetitive motif "HF/SRIG" (label (6), positions 71 - 82); the predicted transmembrane anchor of Vpr required for the ion channel activity of Vpr (label (7), positions 52-79).

Figure 2: Immunological characterization of polyclonal antibodies specific for sVpr$^{1-96}$ by Western blot and immune precipitation.

Rabbits were immunized with sVpr$^{1-96}$ and the resulting serum *R-96* was tested in Western blot (A) and immune precipitation (B). A serial dilution of sVpr$^{1-96}$, 0.01 to 10 ng, was separated in a SDS-PAGE (12.5% acryl aide gel) (A). A similar serial dilution of sVpr$^{1-96}$ was added to human serum and from the mixture sVpr$^{1-96}$ was recovered by immune precipitation using the serum *R-96* followed by separation of the immune precipitates in a SDS-PAGE (B). sVpr$^{1-96}$ was electro-transferred onto PVDF-membranes and the peptide was detected using *R-96* followed by binding to

^{125}I- labeled protein G. The autoradiogram of a two-day exposure is shown in (A) and (B). Positions of the molecular weight standard proteins are indicated on the left, positions of the light (lc) and heavy chain (hc) of immune globulins used for immune precipitation are indicated on the right.

Figure 3: *s*Vpr$^{1-96}$ activates virus replication and increases the number of live cells in cultures of human PBMC.

Cultures of PHA- and IL-2-activated PBMCs were infected with equal infectious doses of the following virus stocks: HIV-1_{NL4-3} (A, B, C), NL4-3(AD8) (D) as well as the *vpu*-deficient mutant NL(AD8)-U_{DEL1} (E), and the *vpr*-deficient mutant NL(AD8)deltaR (F). During the course of the experiment cultures were incubated with 10 nM of *s*Vpr$^{1-96}$ or 10 nM of the control peptide Vpu$^{32-81}$. Virus release is demonstrated as the profile of virus associated RT-activity released into the cell culture supernatant (A,C,D,E,F). (B) shows the number of live cells detected in the cultures of experiment (A).

Figure 4: *s*Vpr$^{1-96}$ activates virus replication of *vpr*-deficient HIV-1 mutant viruses in cultures of primary human monocytes/macrophages isolated from different donors. Parallel cultures of differentiated MDM isolated from three different donors were infected with equal infectious doses of purified virus stocks of the macrophage-tropic virus NL4-3(AD8) as well as the *vpr*-deficient mutant NL(AD8)deltaR. Virus production was followed over a time frame of two months and release of virus associated RT-activity was plotted against time.

Figure 5: 2D ^{1}H TOCSY spectrum.
Mixing time was 110 ms, the spectrum was recorded of a 2 mM solution of *s*Vpr$^{1-96}$ in 1:1 ($^{V}/_{V}$) TFE-d2/H2 at 300°K. The x- and y- axes demonstrating the respective 1D ^{1}H spectra. Enlargements of regions A, B and C are shown in figure 6.

Figure 6: Enlargements of regions of a 2D TOCSY spectrum shown in figure 5 that correlates to interaction of protons between proline H-7 and H-2 of tryptophan residues (A); H-2 and H-4 of histidine residues (B), and epsilon-H and alpha-H of arginine residues (C).

Figure 7: sVpr$^{1-96}$ - HPLC chromatogram and mass spectrum.

Figure 8: sVpr$^{1-47}$ - mass spectrum.

Figure 9: sVpr$^{1-20}$ - mass spectrum.

Figure 10: sVpr$^{21-40}$ - mass spectrum.

WHAT IS CLAIMED IS:

1. Synthetic peptides comprising the regulatory virus protein R (Vpr) of the human immunodeficiency virus type 1(HIV-1).

2. The method of Claim 1, wherein said that peptides are characterized as follows

2.1. as a 96 amino acid long Vpr protein (sVpr$^{1-96}$)

2.2. as a 47 amino acid long N-terminal peptide (sVpr$^{1-47}$)

2.3. as a 49 amino acid long C-terminal peptide (sVpr$^{48-96}$), as well as

2.4. fragments of those peptides thereof, for example

2.4.1. overlapping, approximately 15 amino acid long peptides for epitope-characterization and isoelectric focusing

2.4.2. approximately 20 amino acid long peptides for structural and functional characterization of individual domains of Vpr, especially

2.4.2.1. the peptide sVpr$^{1-20}$, and

2.4.2.2. the peptide sVpr$^{21-40}$.

3. Peptides of claims 1 and 2, wherein said that peptides are characterized as follows

3.1. the 96 amino acid full lenght Vpr-Protein sVpr$^{1-96}$ comprises the sequence:

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser - OH

3.2. the 47 amino acid long N-terminal Peptid sVpr$^{1-47}$

H - Met - Glu - Gln - Ala - Pro - Glu - Asp - Gln - Gly - Pro - Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu

- Ala - Val - Arg - His - Phe - Pro - Arg - Ile - Trp - Leu - His - Asn - Leu - Gly - Gln - His - Ile - Tyr - NH_2

3.3. the 49 amino acid long C-terminal peptide sVpr$^{48-96}$

Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile - Ile - Arg - Ile - Leu - Gln - Gln - Leu - Leu - Phe - Ile - His - Phe - Arg - Ile - Gly - Cys - Arg - His - Ser - Arg - Ile - Gly - Val - Thr - Arg - Gln - Arg - Arg - Ala - Arg - Asn - Gly - Ala - Ser - Arg - Ser-OH

3.4. fragments of those peptides thereof consisting of 15 amino acid long peptides

3.4.1. sVpr$^{11-25}$

Gln - Arg - Glu - Pro - Tyr - Asn - Glu - Trp - Thr - Leu - Glu - Leu - Leu - Glu - Glu

3.4.2. sVpr$^{41-55}$

Asn - Leu - Gly - Gln - His - Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala

3.4.3. sVpr$^{46-60}$

Ile - Tyr - Glu - Thr - Tyr - Gly - Asp - Thr - Trp - Ala - Gly - Val - Glu - Ala - Ile

3.4.4. sVpr$^{56-70}$

Gly- al-Glu-Ala-Ile-Ile-Arg-Ile-Leu-Gln-Gln-Leu-Leu-Phe-Ile

3.5. as the approximately 20 amino acid long peptides

3.5.1. the peptide sVpr$^{1-20}$ in form of

sVpr$^{1-20}$(Asn^{5,10,14})

H-Met - Glu - Gln - Ala - Asn - Glu - Asp - Gln - Gly - Asn - Gln - Arg - Glu - Asn - Tyr - Asn - Glu - Trp - Thr - Leu-NH_2, and

3.5.2. the peptide *s*Vpr$^{21-40}$ in form of

*s*Vpr$^{21-40}$(Asn^{35})

H-Glu - Leu - Leu - Glu - Glu - Leu - Lys - Ser - Glu - Ala - Val - Arg - His - Phe - Asn - Arg - Ile - Trp - Leu - His-NH_2

4. Method for the production of synthetic peptides derived from the regulatory virus proteins R (Vpr) of the human immunodeficiency virus type 1 (HIV-1) according to claims 1 to 3, wherein said that the synthesis of C-terminal Vpr-peptides is performed on a serine resin using a Perkin Elmer synthesizers and all N-terminal peptides are synthesized on a polystyrene polyoxyethylen resin. All peptides were synthesized using FMOC protection group strategy.

5. Method of claim 4, wherein said that at the end of the synthesis protection groups are cleaved off using a cleavage mixture consisting of 95% trifluoracetic acid, 3% triisopropylsilane, and depending on the peptide sequence 2 to 5 % ethandithiol, and the resin was separated.

6. Methods of claims 4 and 5, wherein said that the raw peptides were purified by HPLC chromatography on a column of silica gel using a linear gradient of TFA and water in acetonitrile.

7. Application of synthetic peptides derived from the regulatory virus proteins R (Vpr) of the human immunodeficiency virus type 1 (HIV-1) for therapeutic and diagnostic use.

8. Application of claim 7

8.1. in biological assays

8.1.1. for the development of serological test systems

8.1.2. for the development of Vpr antigen capture ELISA (enzyme linked immune sorbent assays)

8.2. for the generation of antibodies directed against HIV peptide sequences

8.3. for the generation of anti-viral reagents

8.4. for the development of test systems to screen potential Vpr inhibitors.

8.5. for the development of cell culture and animal models to investigate pathological mechanism of Vpr

8.6. for structural analyses of Vpr and its domains

8.7. for application in *in vitro* assembly of viruses and the development of vectors for application in gene therapy.

9. Applications according to claims 7 and 8, wherein said that those proteins are derived from *s*Vpr in which the N-terminal domain is mutated in either one, some, or all of the four proline residues.

10. Application according to claims 7 to 9 for the production of poly- and monoclonal antibodies or antisera specific for Vpr.

11. Application claims 7 to 10 for the production of epitope-different Vpr specific antibodies.

12. Application according to claims 7 to 11 in serological test systems.

13. Application according to claims 7 to 12 in a Vpr antigen (Ag) ELISA.

14. Application according to claims 7 to 13 as standard antigen for the calibration of Vpr-Ag-ELISA techniques.

15. Application according to claims 7 to 8 for the detection and for the estimation of Vpr concentration of viral Vpr in peripheral blood of HIV infected individuals.

16. Application of *s*Vpr proteins according to claims 7 and 8 for *in vitro* test systems for the characterization of Vpr Inhibitors.

17. Application according to claims 7 and 8 for complementation of the function of endogenous viral Vpr in cell cultures infected with *vpr* deficient HIV mutants.

18. Application according to claims 7, 8 and 17 for complementation of the function of endogenous viral Vpr in cultures of primary human lymphocytes infected with *vpr* deficient HIV mutants.

19. Application according to claims 7, 8, 17 18 zur for complementation of the function of endogenous viral Vpr in cultures of primary human monocytes / macrophages infected with *vpr* deficient HIV mutants.

20. Application according to claims 7 to 19 for the characterization of reagents which
a) block the interaction of Vpr with cellular factors, like the glucocorticoid-receptor, transcription factors and other DNA interacting enzymes and factors;
b) block the transcription activating activity of Vpr, and regulate, interfere with or block the activity of Vpr on steroid hormones;
c) regulate, interfere with , or block the transport of Vpr on its own or in complex with other components of the HIV preintegration complex as well as regulate, interfere with, or block the encapsidation of Vpr in budding virus particles during the HIV assembly;
d) regulate, interfere with, or block the Vpr induced cell cycle arrest as well as regulate, interfere with, or block the effect of Vpr on cell differentiation and cell growth;

e) regulate, interfere with, or block the cytotoxic effect of Vpr;

f) regulate, interfere with, or block the ion channel activity of.

21. Application of *s*Vpr proteins according to claims 7 and 8 for *in vivo* test systems that characterize Vpr inhibitors.

22. Application of *s*Vpr proteins according to claims 7 and 8 in animal model studies for the characterization of functions according to claim 20.

23. Application of *s*Vpr proteins according to claims 7 and 8 for the production of concentrated peptide solutions.

24. Application of *s*Vpr proteins according to claims 7, 8 and 23 for the production of Vpr specific inhibitors.

25. Application of *s*Vpr proteins according to claims 7, 8, 21 and 24 for the application of structure stabilizing factors that reduce the flexibility of the N-terminal domain in Vpr.

26. Application according to claim 25, wherein said that those structure stabilizing factors are

a) the UBA2 domain of the DNA repairing enzyme HHR23A which bind to Vpr,

b) Fab fragments of Vpr specific immune globulins,

c) viral factors, especially components of the HIV-1 Gag polyprotein precursor $Pr55^{Gag}$ which interacts with Vpr during the process of Virus assembly; or

d) the human glycocorticoid receptor or components thereof.

27. Application of *s*Vpr proteins according to claim 7 for *in vitro* assembly of retroviral preintegration complexes.

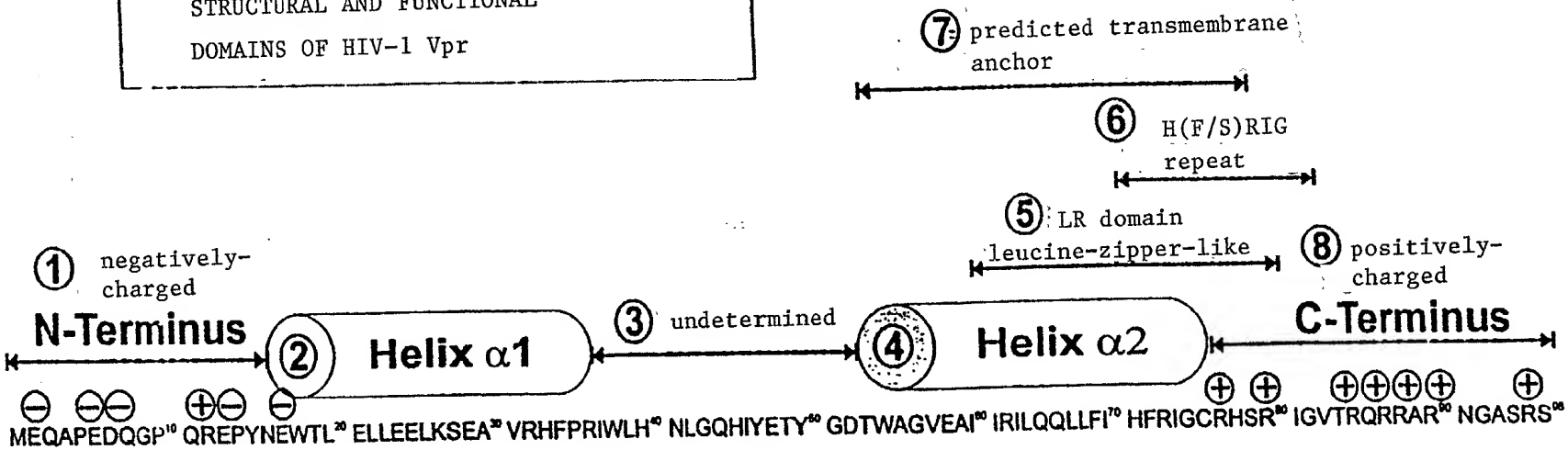
28. Application of *s*Vpr proteins according to claims 7, 8 and 27 in *in vitro* or *in vivo* application of gene transfer methods.

29. Application of *s*Vpr proteins according to claims 7, 8 and 28 for transfection, integration into chromosomal and episomale host DNA or any other gene transfer methods in eukaryotic cells.

30. Application of *s*Vpr proteins according to claims 7, 8 and 28 for gene transfer using *in vitro* synthesized and / or manipulated genes and fragments thereof for the transfer into cells, tissues, and organisms, and any application for the purpose of gene therapy.

FIGURE 1

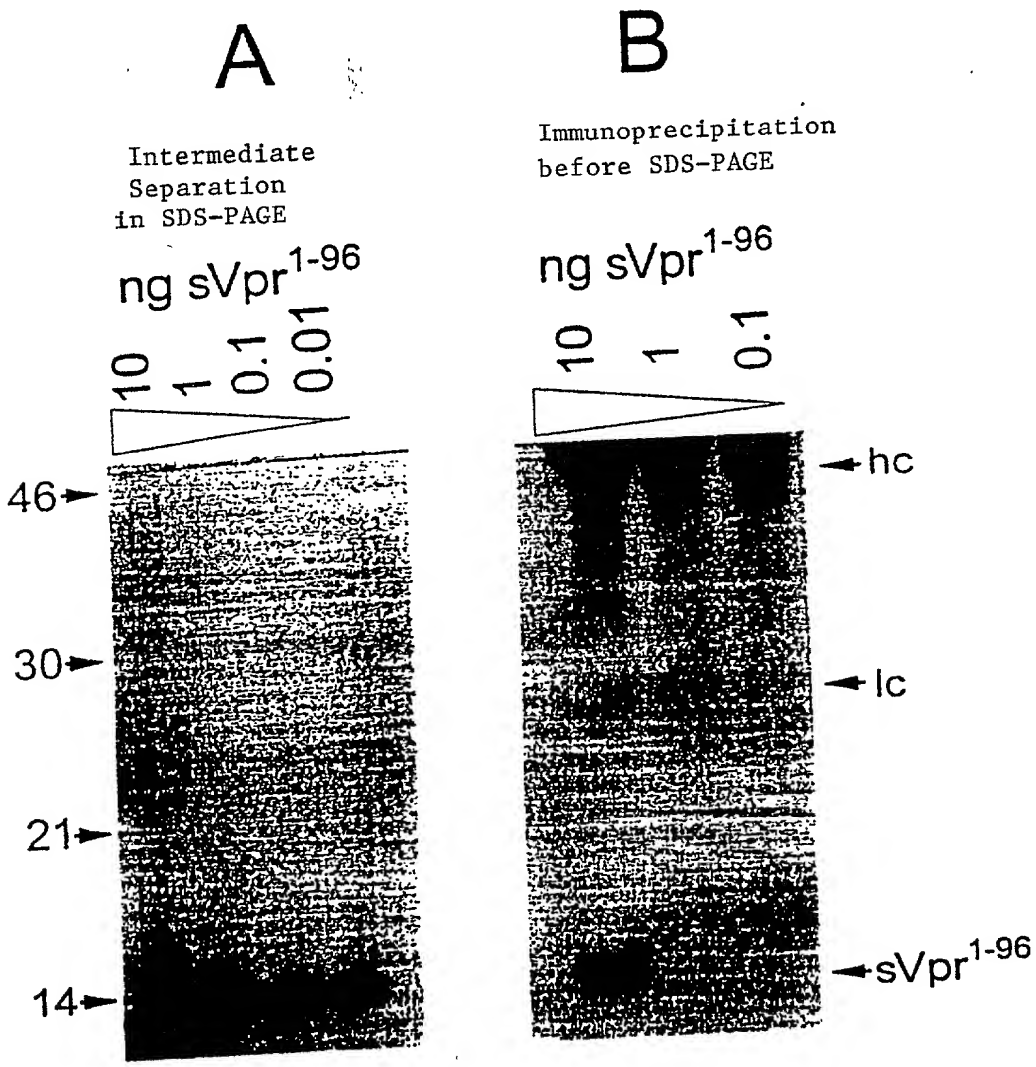
STRUCTURAL AND FUNCTIONAL
DOMAINS OF HIV-1 Vpr



Sequence from HIV-1_{HL4-3}

DOMAINS	FUNCTIONS
②④	Entry of Vpr in virus particle
②④⑤	Transport into cell nucleus
②②④	Cell cycle arrest / cell differentiation
①②③	Oligomerization
⑥⑧	Cytotoxicity and induction of apoptosis
④⑧	Ion channel activity
⑤⑧	Transcription activation and interaction with Sp1
④⑤	Regulation of glucocorticoid receptors

FIGURE 2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Separation
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1
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46
30
21
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Immunoprecipitation
before SDS-PAGE
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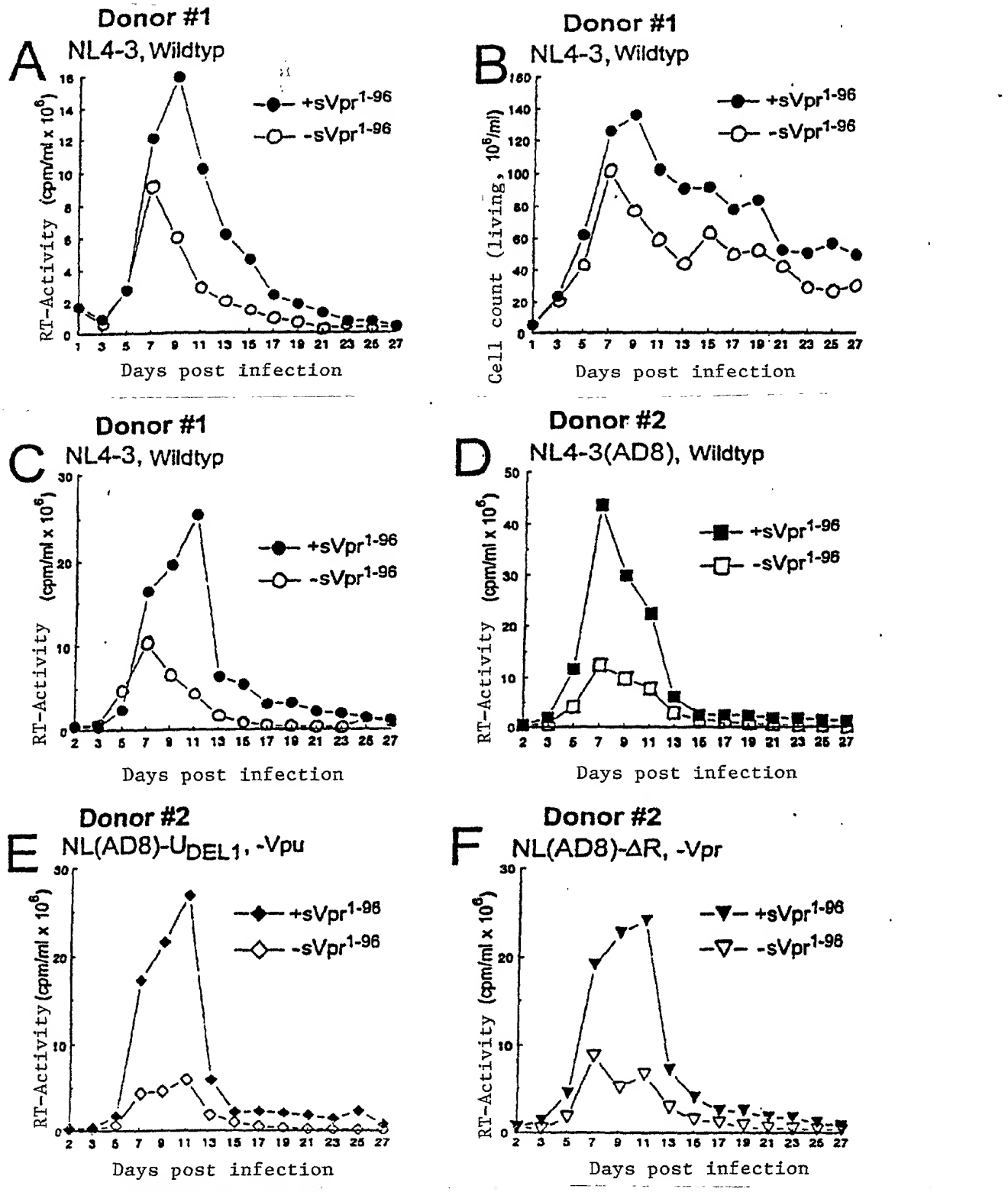
10
1
0.1
hc
lc
sVpr1-96

FIGURE 3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A NL4-3, Wildtyp
RT-Activity (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Days post infection
Donor #1
B NL4-3, Wildtyp
Cell count (living, 10^5/ml)
+sVpr1-96
-sVpr1-96
Days post infection
Donor #1
C NL4-3, Wildtyp
RT-Activity (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Days post infection
Donor #2
D NL4-3(AD8), Wildtyp
RT-Activity (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Days post infection
Donor #2
E NL(AD8)-U_DEL1, -Vpu
RT-Activity (cpm/ml x 10^6)
+sVpr1-96
-sVpr1-96
Days post infection
Donor #2
F NL(AD8)-ΔR, -Vpr
RT-Activity (cpm/ml x 10^6)
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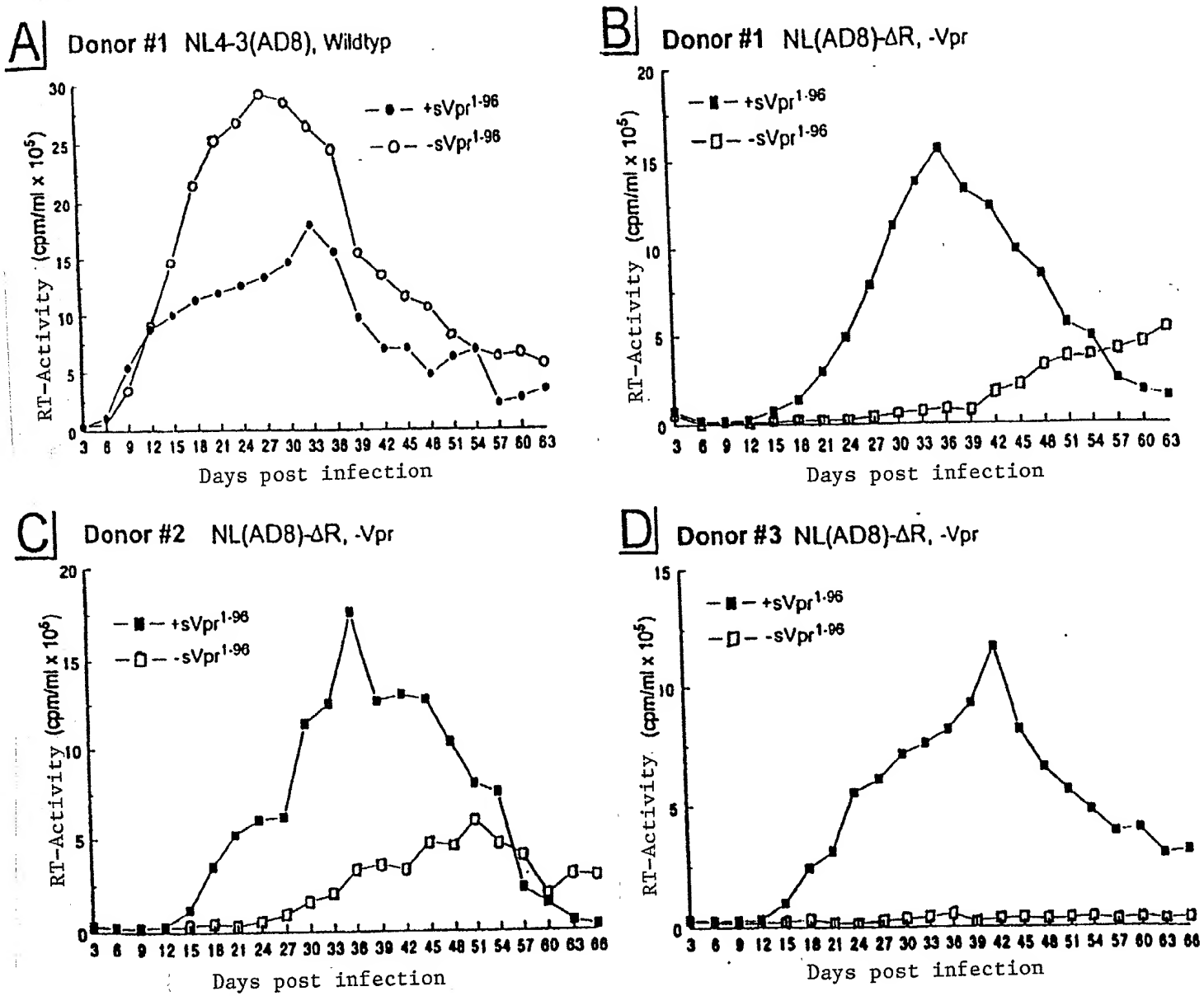
-sVpr1-96
Days post infection

FIGURE 4


A
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+sVpr1-96
-sVpr1-96
RT-Activity (cpm/ml x 10^5)
Days post infection
B
Donor #1 NL(AD8)-ΔR, -Vpr
+sVpr1-96
-sVpr1-96
RT-Activity (cpm/ml x 10^5)
Days post infection
C
Donor #2 NL(AD8)-ΔR, -Vpr
+sVpr1-96
-sVpr1-96
RT-Activity (cpm/ml x 10^5)
Days post infection
D
Donor #3 NL(AD8)-ΔR, -Vpr
+sVpr1-96
-sVpr1-96
RT-Activity (cpm/ml x 10^5)
Days post infection

FIGURE 5

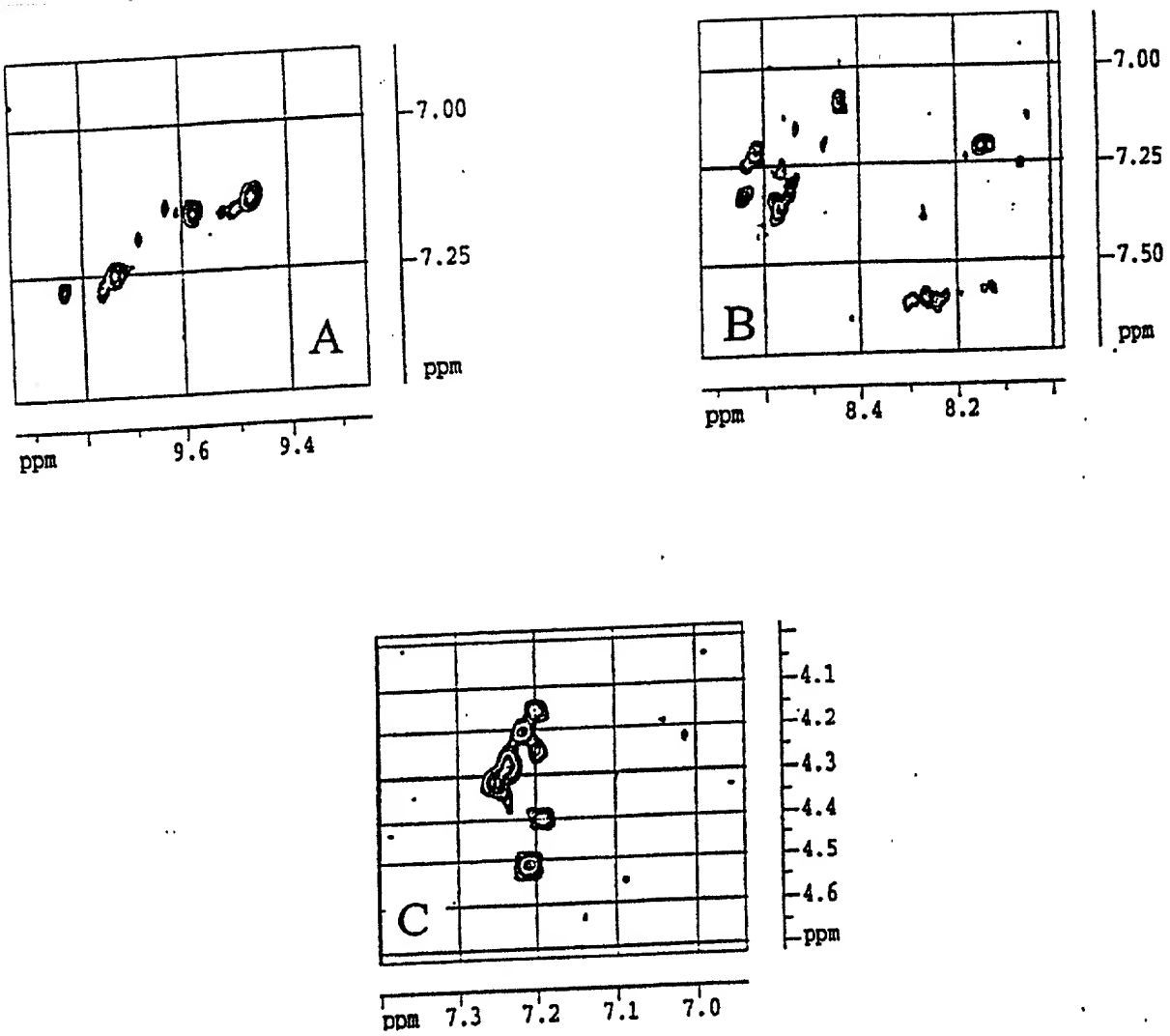
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FIGURE 6

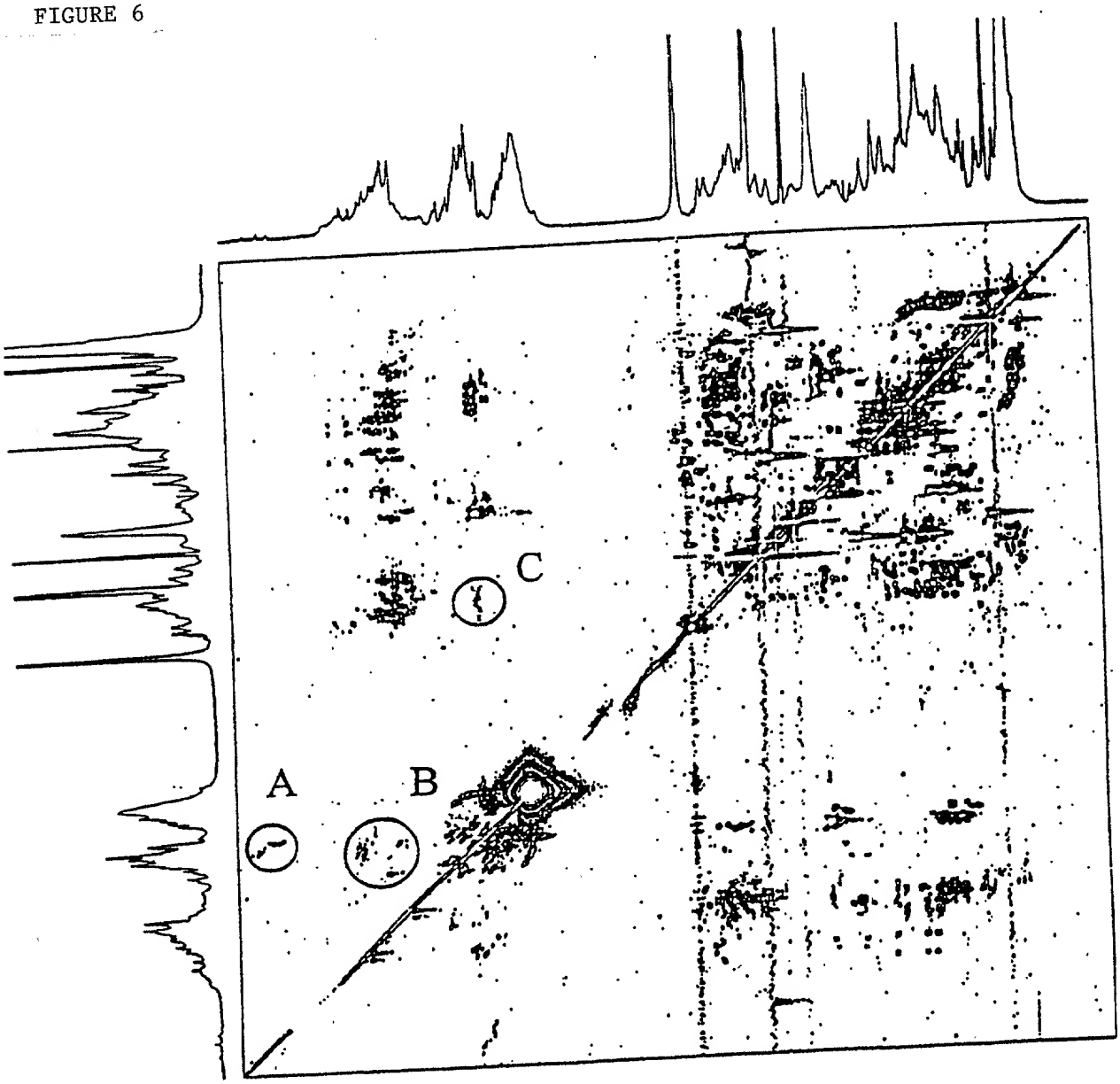
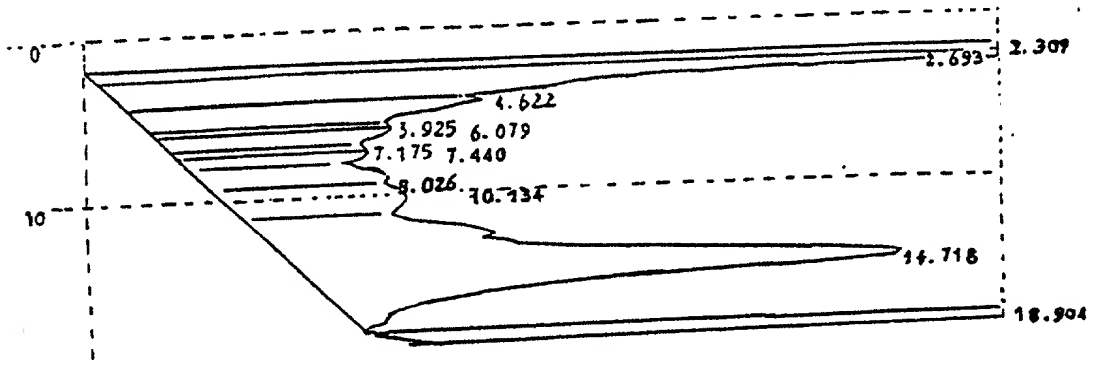


FIGURE 7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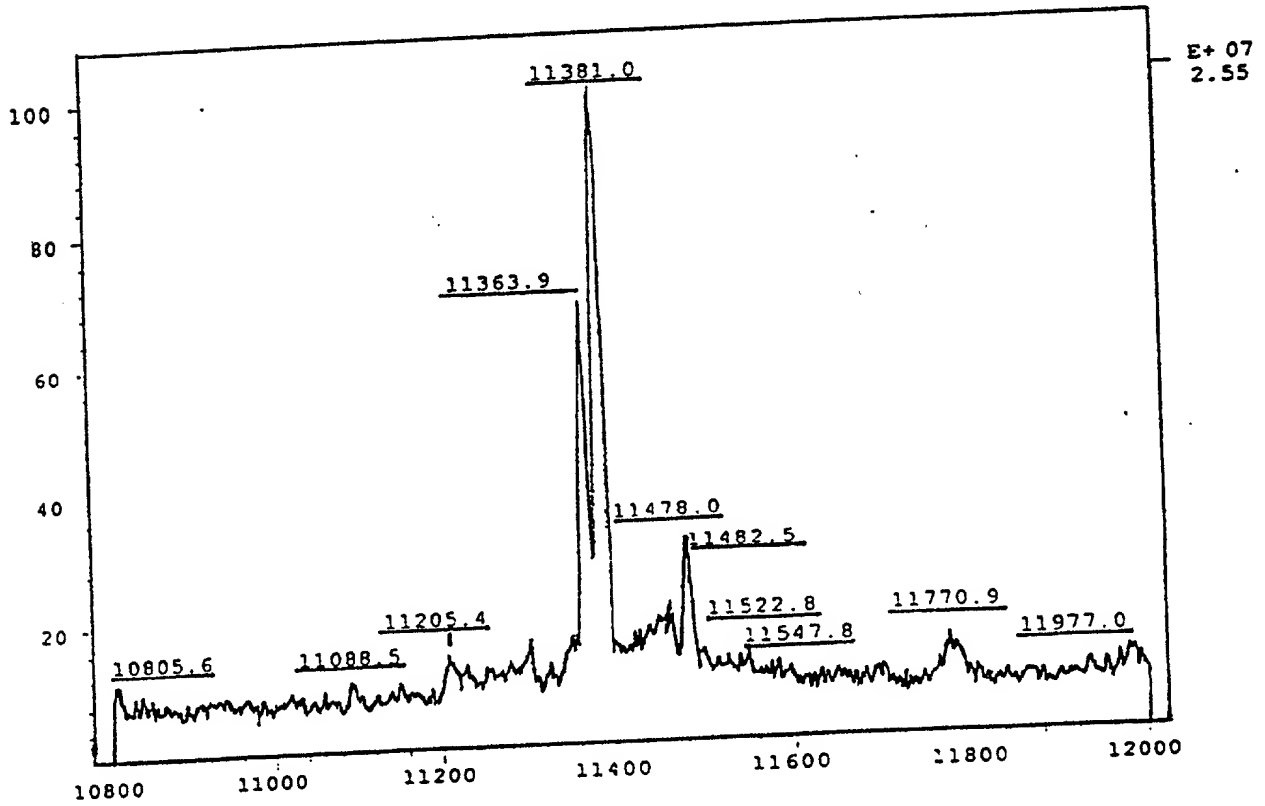
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40
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11200
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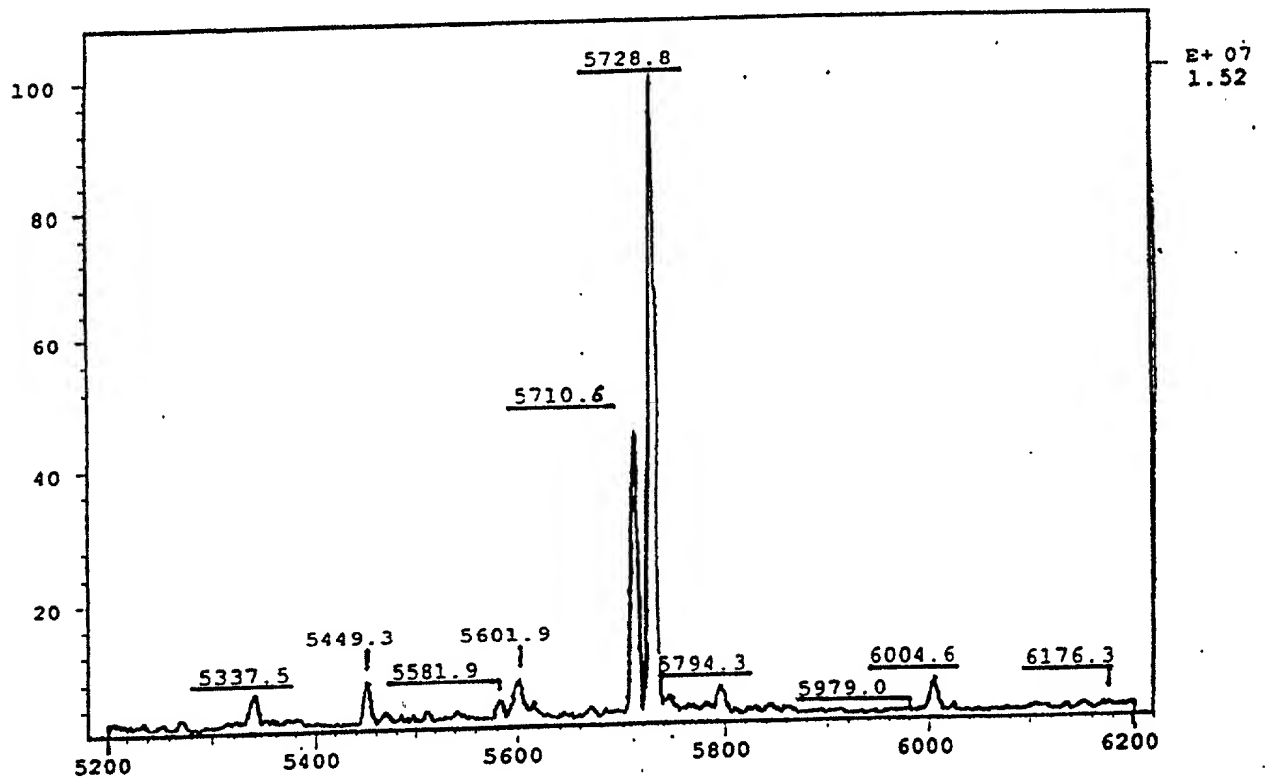
FIGURE 8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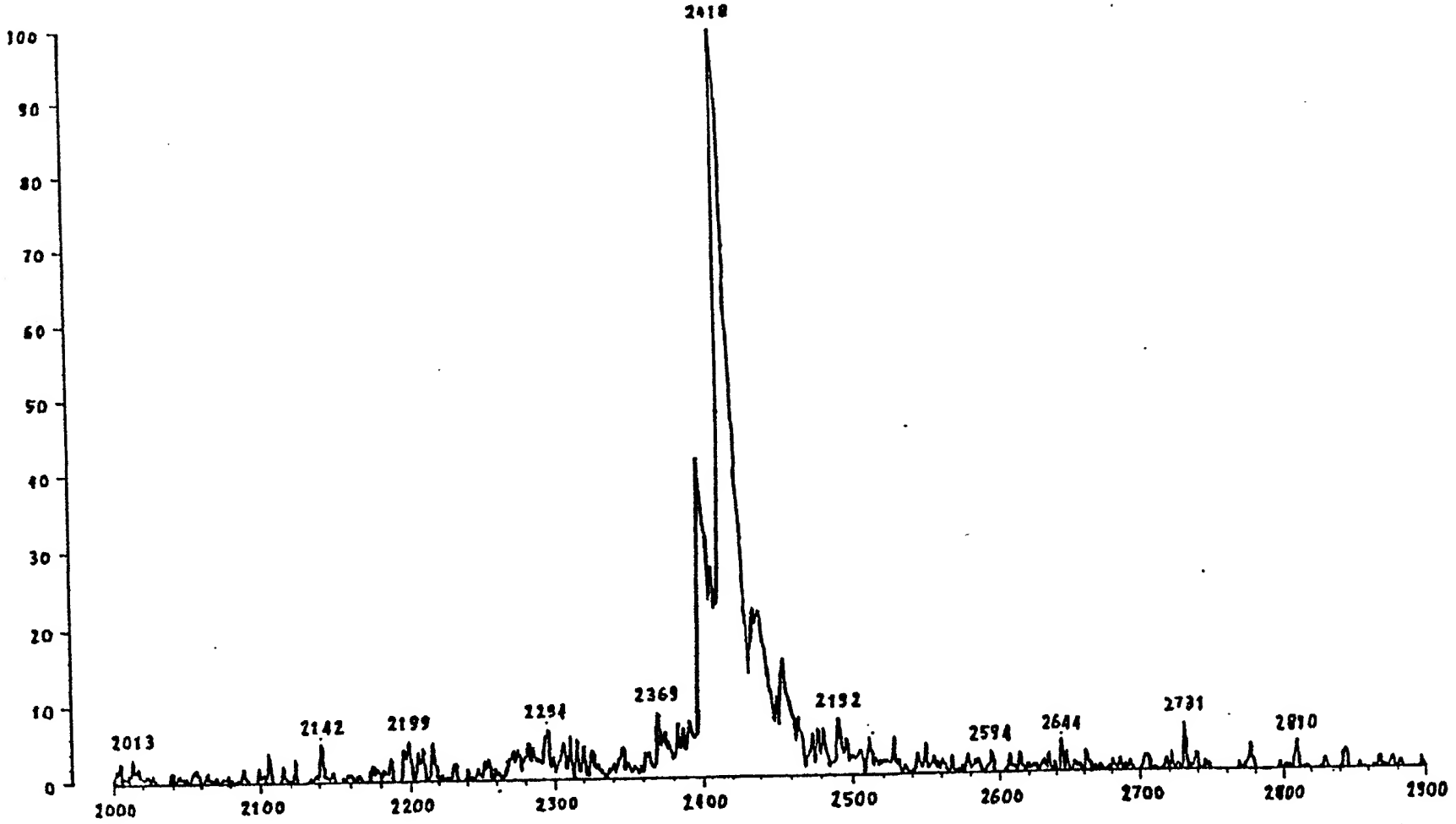
5200
5400
5600
5800
6000
6200

FIGURE 9



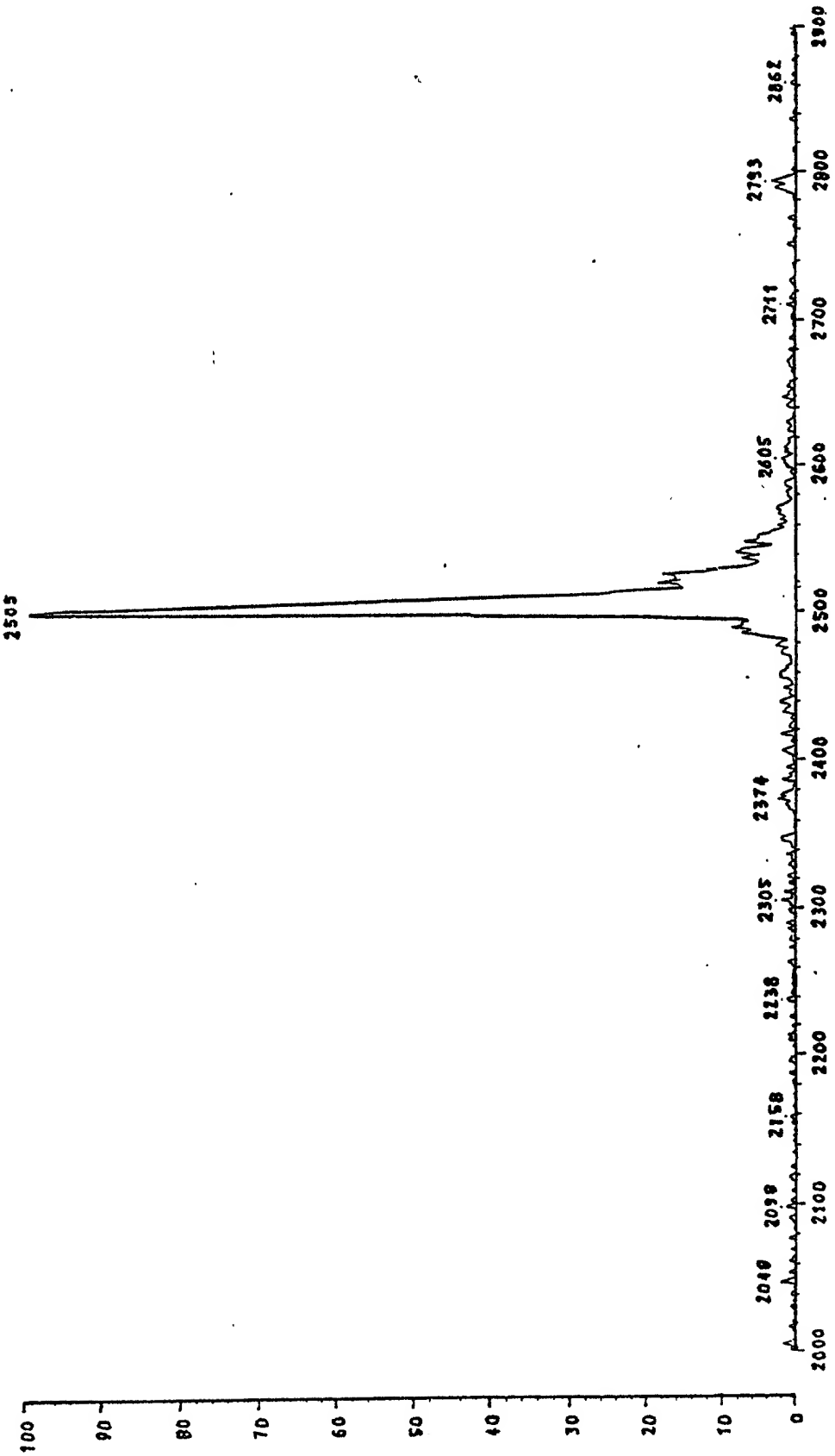


FIGURE 10

GATES & COOPER LLP

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND THE UTILIZATION THEREOF

The specification of which:

a. ☐ is attached hereto.

b. ☒ was filed on February 19, 2000 as PCT International Application Number PCT/DE00/00525, which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT application having a filing date before that of the application on the basis of which priority is claimed:

a. ☐ no such applications have been filed.
b. ☒ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	**APPLICATION NUMBER**	**DATE OF FILING (day, month, year)**	**DATE OF ISSUE (day, month, year)**
GERMANY	199 08 752.0	19 FEB 99	
GERMANY	199 08 766.0	19 FEB 99	
OTHER FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	**APPLICATION NUMBER**	**DATE OF FILING (day, month, year)**	**DATE OF ISSUE (day, month, year)**

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or 365(c) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose

material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. PARENT APPLICATION OR PCT PARENT NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)

I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

George H. Gates	Registration No. 33,500
Victor G. Cooper	Registration No. 39,641
Karen S. Canady	Registration No. 39,927
William J. Wood	Registration No. 42,236
Jason S. Feldmar	Registration No. 39,187
Bradley K. Lortz	Registration No. 45,472

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Gates & Cooper LLP to the contrary.

Please direct all correspondence in this case to the firm of Gates & Cooper LLP at the address indicated below:

CUSTOMER NUMBER 22462
Gates & Cooper LLP
Howard Hughes Center
6701 Center Drive West, Suite 1050
Los Angeles, CA 90045
(310) 641-8797

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(1)	**Full Name Of Inventor**	**Family Name** SCHUBERT	**First Given Name** Ulrich	**Second Given Name**
	Residence & Citizenship	**City** Bethesda	**State or Foreign Country** Maryland	**Country of Citizenship** GERMANY
	Post Office Address	**Post Office Address** 4616 Maple Avenue	**City** Bethesda	**State & Zip Code/Country** Maryland 30814 / U.S.A.
Signature of Inventor(1):				**Date:**
(2)	**Full Name Of Inventor**	**Family Name** HENKLEIN	**First Given Name** Peter	**Second Given Name**
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	Post Office Address	**Post Office Address** Schulze-Boysen-Strasse 25	**City** Berlin	**State & Zip Code/Country** D-10365 / Germany
Signature of Inventor(2):				**Date:**
(3)	**Full Name Of Inventor**	**Family Name** WRAY	**First Given Name** Victor	**Second Given Name**
	Residence & Citizenship	**City** Wolfenbuttel	**State or Foreign Country** Germany	**Country of Citizenship** UNITED KINGDOM
	Post Office Address	**Post Office Address** Elbinger Strasse 6	**City** Wolfenbuttel	**State & Zip Code/Country** D-38302 / Germany
Signature of Inventor(3): Victor Wray				**Date:** 3.9.01

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Signature of Inventor(2): [signature]				Date: 09/05/2007
(3)	Full Name Of Inventor	Family Name WRAY	First Given Name Victor	Second Given Name
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(1) Full Name Of Inventor	Family Name SCHUBERT	First Given Name Ulrich	Second Given Name
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Signature of Inventor(1): Ulrich Schubert			Date: 09-05-01
(2) Full Name Of Inventor	Family Name HENKLEIN	First Given Name Peter	Second Given Name
Residence & Citizenship	City Berlin	State or Foreign Country Germany DEX	Country of Citizenship GERMANY
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Signature of Inventor(2):			Date:
(3) Full Name Of Inventor	Family Name WRAY	First Given Name Victor	Second Given Name
Residence & Citizenship	City Wolfenbuttel	State or Foreign Country Germany DEX	Country of Citizenship UNITED KINGDOM
Post Office Address	Post Office Address Elbinger Strasse 6	City Wolfenbuttel	State & Zip Code/Country D-38302 / Germany
Signature of Inventor(3):			Date:

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) it establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) it refutes, or is inconsistent with, a position the applicant takes in:

(i) opposing an argument of unpatentability relied on by the Office, or

(ii) asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) each inventor named in the application:

(2) each attorney or agent who prepares or prosecutes the application; and

(3) every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

NONPROFIT ORGANIZATION

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. 1.9(e) AND 1.27(d)) – NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: The J. David Gladstone Institute
ADDRESS OF ORGANIZATION: P.O. Box 419100
San Francisco, California 94141-9100

TYPE OF NONPROFIT ORGANIZATION:

a) ☐ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

b) ☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3))

c) ☒ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE_____)
(CITATION OF STATUTE_____)

d) ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA

e) ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE_____)
(NAME OF STATUTE_____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in regard to the invention, entitled: **SYNTHETIC PEPTIDE OF REGULATORY VIRUS PROTEIN R (VPR) OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) AND THE UTILIZATION THEREOF** by inventor(s) Ulrich Schubert, Peter Henklein and Victor Wray described in:

☒ U.S. national stage patent application Serial No. 09/913,927, corresponding to International Application No. PCT/DE00/00525 which was filed on February 19, 2000.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37b C.F.R. 1.9(c) or by any concern which would not qualify as a small business concern under 37 C.F.R. 1.9(d) or a nonprofit organization under 37 C.F.R. 1.9(e). ***NOTE:** Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. 1.27)

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS ☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as small entity is no longer appropriate. (37 C.F.R. 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that

willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereof, or any patent to which this verified statement is directed.

NAME: Joan V. Bruland

TITLE: Intellectual Property Counsel

ADDRESS: THE J. DAVID GLADSTONE INSTITUTES
P.O. Box 419100
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SIGNATURE: Joan V. Bruland

DATE: 9/4/01

United States Patent & Trademark Office

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Drawing figures 2, 5, 6 are very dark.